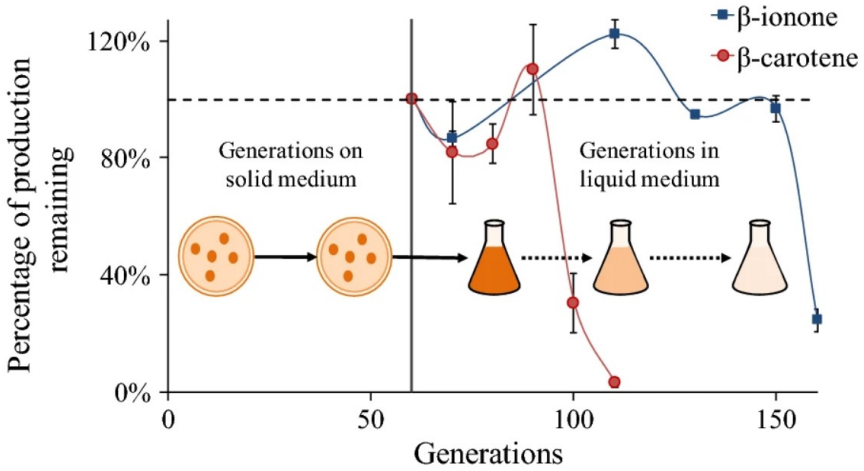
Washington U/ U. Delaware ABF DFO

Lawrence Berkeley National Laboratory
Pacific Northwest National Laboratory

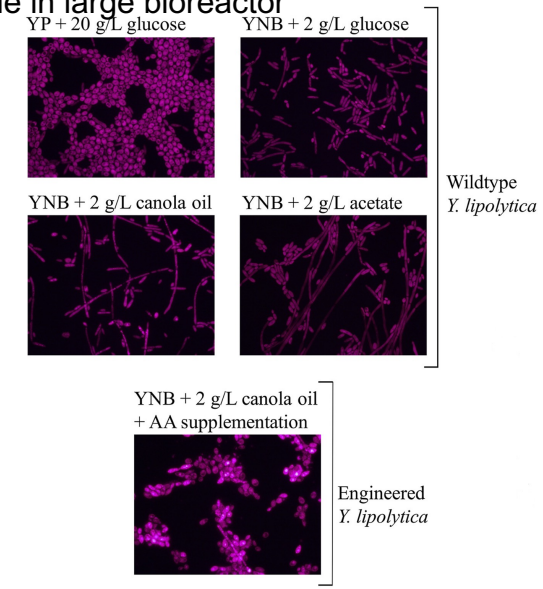
FY23 – FY24

Project Overview

- Yarrowia lipolytica is an oleaginous yeast that is a promising biomanufacturing chassis
- However, phenotypic changes and titer instability are encountered during scale-up
  - Engineered β-carotene-producing strain exemplifies this issue in large bioreactor



β-carotene producing strain was developed as a platform for the production of b-ionone as part of a collaboration between CoPI Tang and Arch Innotek, a St. Louis biotech start-up. Czajka et al., 2018: https://doi.org/10.1186/s12934-018-0984-x



Effect of medium compositions on cell morphology during growth phase Worland et al., 2020: https://doi.org/10.1016/j.mec.2020.e00130

Project Overview

Yinjie Tang (expert in ^{13}C metabolic analysis and yeast fermentations) and Mark Blenner (expert in metabolic engineering and synthetic biology of Y. lipolytica) submitted a DFO proposal in FY20 to answer the following questions.

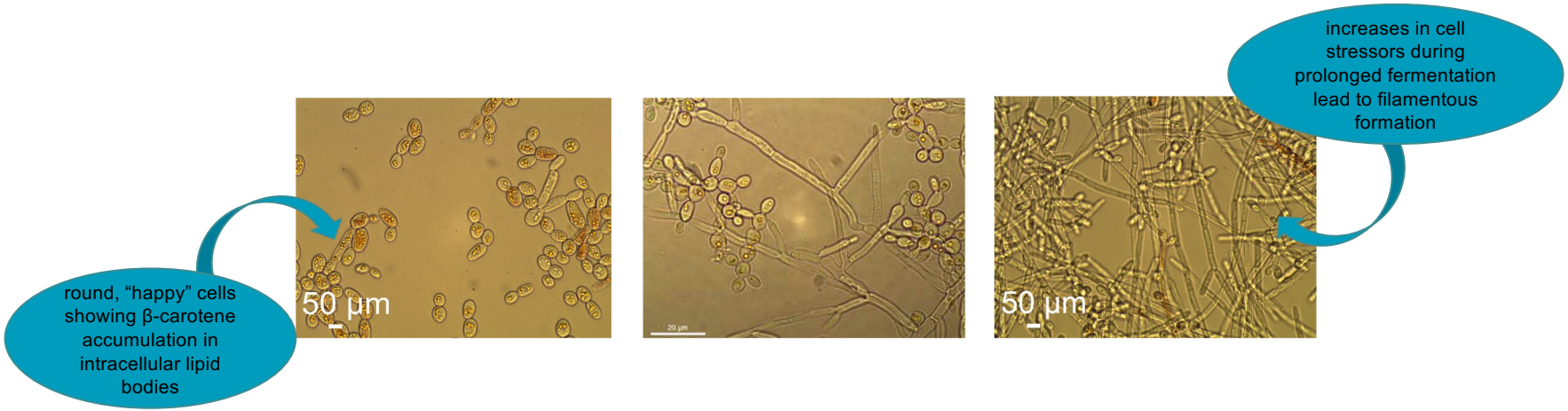
Questions:

1) Is productivity loss caused by genetic (i.e., mutations) or epigenetic changes during extended growth?
2) Does the genetic mutation or epigenetic change rate increase at specific locations (e.g., engineered genes) during extended growth? What is the buffering capability? https://pubmed.ncbi.nlm.nih.gov/32925942/
3) How does metabolic stress increase the genetic mutation or epigenetic change rate, and how do bioreactor conditions influence these changes?

1 - Approach

- Investigate the nature of titer instability using β-carotene strain as a model and understand more broadly the factors that lead to cellular heterogeneity during cell line development and scaleup

- Understanding the molecular basis of titer decline during scaleup requires integrating long duration bioreactor operation with multiple omics experiments



1 – Approach

Task	Hypothesis	Approach
1	Loss of productivity is caused by accumulation of mutations prolonged generations of cell growth	Conduct continuous fermentations at 2L scale to at least 25 generations of wild type and β-carotene engineered Y. lipoytica. Deep sequence genomes at different generations; biomass, exudate, & titer analyses
2	Overall mutation rate and β-carotene pathway mutation rate are influence by the culture conditions and metabolic burden	Conduct continuous fermentations at 2L scale to at least 25 generations of β-carotene engineered Y. lipoytica under stressful conditions (low O2, low nutrients, pH) Deep sequence genomes at different generations; biomass, exudate, titer analyses
3	Metabolic burden and culture conditions alter the overall and local mutagenesis rate and affect phenotypical changes	From fermentations in Task 2, perform RNASeq, targeted proteomics and targeted metabolomics to determine the mechanisms

1 – Approach

Timelines

Fermentation at LBNL:

1. ABPDU Engineers enabled Chemostat software and process at 2L scale in Spring 2021
2. Alyssa Worland, graduate student from Yinjie Tang Lab, was hired as an EBRC intern in late-May 2021 and initiated first fermentation campaign mid-June 2021
3. All fermentation campaigns will be completed by late-April 2023.

Proteomics and Metabolomics at PNNL:

1. First round of proteomics/ metabolomics to be completed by late-June 2023
2. All proteomics/ metabolomics expected to be completed by late-September 2023

Sequencing and Analysis at University of Delaware and Washington University:

1. All data expected to be collected by late-September 2023
2. All analyses to be completed by late-December 2023

2 – Progress and Outcomes

Development of dual 2L chemostat system for continuous fermentation:

2 – Progress and Outcomes

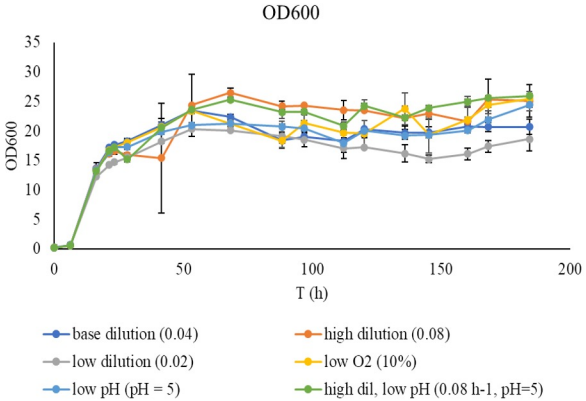
Development of semi-continuous fermentation system in ambr250 HT:

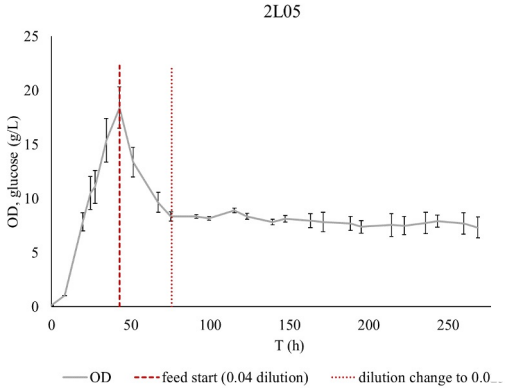
2 – Progress and Outcomes

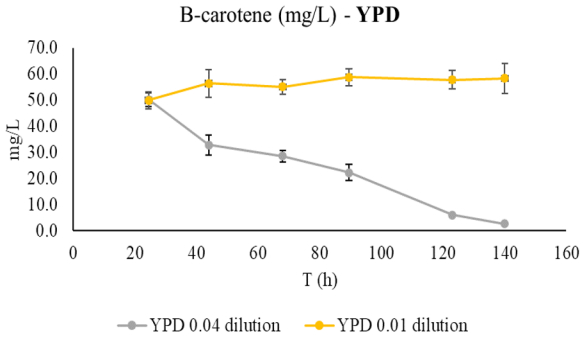
Fermentation	Date	Conditions	Outcome
2L_01	6/15	Single continuous 2L, trial run	Short run due to reactor draining, discovered issue with chemostat system
2L_02	6/28	Single continuous 2L, trial run 2	Solved issue with chemostat system, gathered data on fermentation trends
ambr_01	7/18	Defined and rich media, high/low dilution rate	Observed greater strain instability in defined medium, observed production loss over significantly fewer generations than 2L fermentation
2L_03	8/1	Dual continuous 2L, high/low dilution rate	Developed solutions for foaming issues re-seeding reactor Observed production loss of 82% over 28 generations, and 94% over 10 generations
2L_04	9/3	Dual continuous 2L, high/low oxygen	Observed strange mixing dynamics in high oxygen, potentially due to filamentous growth. One reactor became contaminated.
ambr_02	9/29	Dilution rate, oxygen study, and pH	Observed production loss across all conditions
2L_05	12/01	continuous 2L; oxygen and shake flask study	Low oxygen condition, duplicates Shake flask passaging of same seed (80 gen)

2 – Progress and Outcomes

Task 1: Hypothesis 1: Loss of productivity is caused by accumulation of mutations prolonged generations of cell growth







Work completed

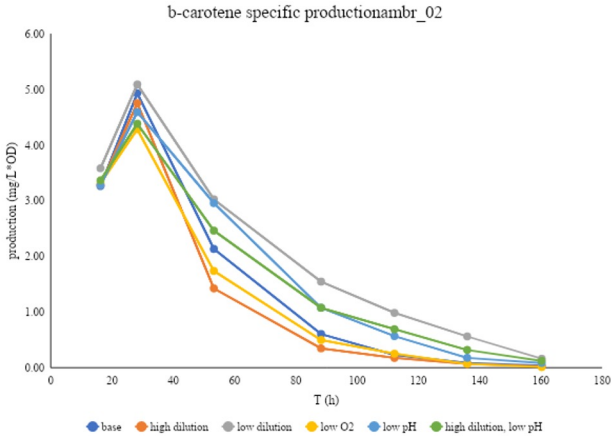
- Conducted continuous cultivation for 16 days in 2L system; 8 days in ambr250
  - demonstrated stability in fermentation with consistent OD measurements
- Product titer loss is observed over increased generations

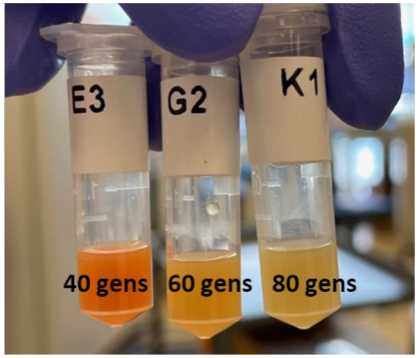
Work ongoing

- Genome sequencing to identify whether production loss is corresponding to obvious genetic mutations

2 – Progress and Outcomes

Task 2: Hypothesis 2: Overall mutation rate and β-carotene pathway mutation rate are influenced by the culture conditions and metabolic burden





Work completed

- Examined several different cultivation conditions using the ambr250 semi-continuous system
- Observed titer loss differed by condition as well as cultivation system (2L vs ambr250)
- Performed shake-flask passaging study as a different method of increasing strain generations

Work ongoing

- Genome sequencing to identify whether production loss is corresponding to obvious genetic mutations

2 – Progress and Outcomes

Task 3: Hypothesis 3: Metabolic burden and culture conditions alter the overall and local mutagenesis rate and affect phenotypical changes

Determine the mechanism by which metabolic burden and culture conditions alter the overall and local mutagenesis rate

Dilution rate	Oxygen levels	Media composition	pH
High vs. low feed/drain rates	High vs. low aeration and agitation rates	Minimal vs. rich media	Controlled vs. non-controlled or standard vs. low

Fermentation analysis	Genome sequencing	Metabolomics, proteomics	Transcriptomics
LBNL - biomass/titer/ overflow/flow cyt	U Delaware	PNNL	U Delaware

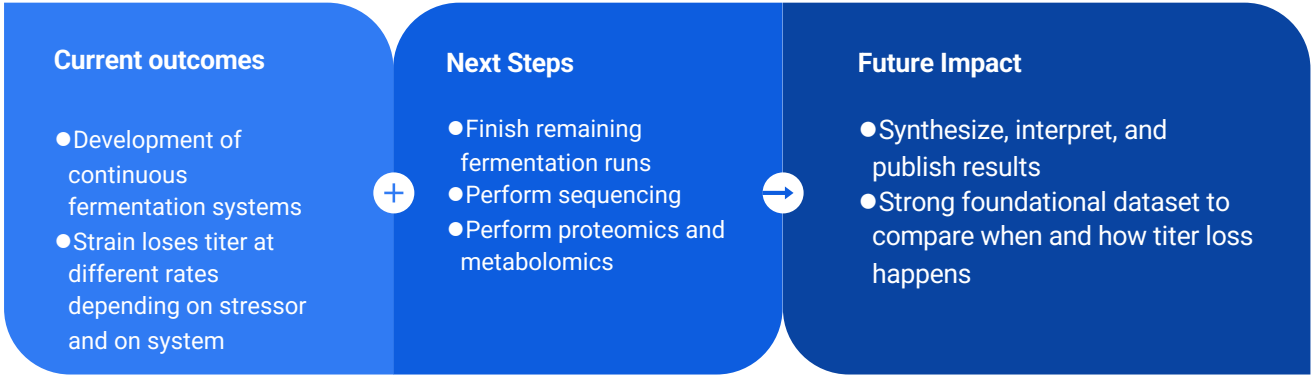
<u>Work completed</u>

- From task 2, fermentations under varying culture conditions (e.g. oxygen limitation)

<u>Work ongoing</u>

- Metabolomics and proteomics of time course samples to identify metabolic shifts and rate limiting enzymes

3 – Impact


Current outcomes
Development of continuous fermentation systems
Strain loses titer at different rates depending on stressor and on system
Next Steps
Finish remaining fermentation runs
Perform sequencing
Perform proteomics and metabolomics
Future Impact
Synthesize, interpret, and publish results
Strong foundational dataset to compare when and how titer loss happens

Quad Chart Overview

Timeline
- *Project Start Date: 10/01/2021*
- *Project End Date: 09/30/2023*

	FY23 Costed	Total Award
DOE Funding	*(10/01/2021 – 9/30/2022)* *250K*	*(negotiated total federal share)* *500K*
Project Cost Share *Only fill out if applicable.	100K	125K

TRL at Project Start: 3 - 4
TRL at Project End: 5 - 7

Project Goal

To link macroscopic causes (e.g., oxygen level, mixing, and shear stresses) to microscopic effects (genetic stability and intracellular metabolism) in a concerted way. Obtain time-course omics information and monitor physiological responses from Yarrowia β-carotene producer under optimal and suboptimal chemostat fermentations.

End of Project Milestone

Identification of optimal gene expression levels for rate limiting enzymes as the targets for suppressing genetic drift and metabolic shifts during fermentation scale up, and clear strain engineering strategies to be implemented and tested in scaleup in a future project.

Funding Mechanism:

Agile Biofoundry DFO FY20

Project Partners*
- Lawrence Berkeley National Laboratory
- Pacific Northwest National Laboratory